November 2005

Separate Functions of Gelsolin Mediate Sequential Steps of Collagen Phagocytosis

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Publisher URL: http://dx.doi.org/10.1091/mbc.E05-07-0648

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Abstract

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Molecular Biology of the Cell
Vol. 16, 5175–5190, November 2005

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Submitted July 19, 2005; Revised August 8, 2005; Accepted August 12, 2005
Monitoring Editor: Anthony Bretscher

Collagen phagocytosis is a critical mediator of extracellular matrix remodeling. Whereas the binding step of collagen phagocytosis is facilitated by Ca^{2+}-dependent, gelsolin-mediated severing of actin filaments, the regulation of the collagen internalization step is not defined. We determined here whether phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] regulation of gelsolin is required for collagen internalization. In gelsolin null fibroblasts transfected with gelsolin severing mutants, actin severing and collagen binding were strongly impaired but internalization and actin monomer addition at collagen bead sites were much less affected. PI(4,5)P2 accumulated around collagen during internalization and was associated with gelsolin. Cell-permeable peptides mimicking the PI(4,5)P2 binding site of gelsolin blocked actin monomer addition, the association of gelsolin with actin at phagosomes, and collagen internalization but did not affect collagen binding. Collagen beads induced recruitment of type 1 γ phosphatidylinositol phosphate kinase (PIPK1γ661) to internalization sites. Dominant negative constructs and RNA interference demonstrated a requirement for catalytically active PIPK1γ661 for collagen internalization. We conclude that separate functions of gelsolin mediate sequential stages of collagen phagocytosis: Ca^{2+}-dependent actin severing facilitates collagen binding, whereas PI(4,5)P2-dependent regulation of gelsolin promotes the actin assembly required for internalization of collagen fibrils.

INTRODUCTION

Collagen is the principal structural protein of the extracellular matrix and is continuously synthesized and degraded by fibroblasts throughout the lifetime of mammals. Intracellular degradation of collagen by phagocytosis is an essential processes for connective tissue homeostasis (Everts *et al*., 1996). Although collagen phagocytosis has been extensively studied in the context of matrix turnover, the mechanisms that regulate fibroblast-mediated intracellular degradation of collagen are poorly understood.

Phagocytosis of collagen fibrils is initiated by binding through $\alpha 2\beta 1$ integrins in avidly phagocytic fibroblasts (Lee *et al*., 1996; Arora *et al*., 2000). Binding is followed by extension of actin-rich pseudopods around fibrils and later, by fibril engulfment (Melcher and Chan, 1981). Extension of the plasma membrane around fibrils is mediated by actin polymerization, a process requiring the generation and uncapping of actin barbed ends. Gelsolin is an actin binding protein that regulates actin filament length by severing preexisting filaments and by capping the fast-growing end (or both). These processes are regulated by both Ca^{2+} and phosphoinositides (Janmey *et al*., 1985; Yin, 1987; Kwiatkowski *et al*., 1989). There is substantial evidence for multifunctional roles of gelsolin in various cell functions, including motility and phagocytosis (Azuma *et al*., 1998; Sun *et al*., 1999) but the contributions of the severing and capping functions of gelsolin in integrin-dependent processes have not been defined.

Detailed studies of gelsolin structure using proteolytic fragments (Kwiatkowski *et al*., 1989; Kwiatkowski, 1999), recombinant truncations (Way *et al*., 1989), and crystallography (Burtnick *et al*., 1997) have shown that two different functions of gelsolin (severing and capping/uncapping) and their regulation require cooperative interactions of the G1–G6 domains. The severing function of gelsolin is fully expressed in the amino-terminal G1–3 segments, whereas segments G4–6 have no severing activity; however, they do confer calcium regulation on severing function (Kwiatkowski *et al*., 1985, 1989; Way *et al*., 1989). Considerable evidence suggests that segment G2 binds to the side of the actin filaments, thus facilitating intercalation of G1 between actin subunits in the filament and resulting in disruption of actin-actin contacts.

Because reversibility of actin–gelsolin associations is achieved not simply by reducing Ca^{2+}, it is notable that phosphoinositides, particularly phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2], can dissociate gelsolin from actin filaments in vitro (Janmey *et al*., 1987) and can inhibit filament severing by gelsolin. Studies of recombinant gelsolin deletion mutants have identified a 10-amino acid sequence responsible for the phosphoinositide binding activity of gelsolin. The peptide QRLFQVKGRR, derived from segment

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05–07–0648) on August 24, 2005.

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G2 (residues 160–169), competes with intact gelsolin for binding to phosphoinositides (Cunningham *et al*., 2001), supporting previous work that PI(4,5)P2 induces actin assembly by dissociating gelsolin-caps from actin filament ends (Janmey and Stossel, 1989). Currently, the role of PI(4,5)P2 in mediating collagen phagocytosis is not defined; the identity and localization of the enzymes that generate PI(4,5)P2 in response to collagen phagocytosis are also not defined. We considered that PI(4,5)P2, as a critical regulator of gelsolin function, may impact collagen phagocytosis.

PI(4,5)P2 is largely generated from PI(4)P and also from PI(5)P by type 1 phosphatidylinositol 5-phosphate kinases [PIP(5)K1] and phosphatidylinositol 4-phosphate kinases (PIP4K1), respectively. Type 1 phosphatidylinositol-4-phosphate (PIP) kinase isoforms have specific and distinct subcellular localizations, providing a means for local generation of PI(4,5)P2. Thus, PIPK1α, PIPK1β, and PIPK1γ have been localized to nuclei, perinuclear regions, and focal contacts, respectively (Loijens and Anderson, 1996; Ling *et al*., 2002). The C termini of the type 1 kinases exhibit divergent sequences; conceivably, specific C-terminal sequences confer distinct functions. The PIPKIγ mRNA transcripts are alternatively spliced, giving rise to PIPK1γ635 and PIPK1γ661 isoforms that differ by a 26-amino acid carboxy-terminal extension (Ishihara *et al*., 1998). Type 1γ661 phosphatidylinositol phosphate kinase (PIPK1γ661), but not PIPK1γ635, is targeted to focal adhesions and interacts with the head domain of talin, thereby regulating turnover of focal adhesions by blocking β-integrin binding (Di Paolo *et al*., 2002; Ling *et al*., 2002, 2003). In view of these findings, we considered that PIPKI isoforms may play specific roles in the local generation of PI(4,5)P2 and regulation of gelsolin involved in collagen phagocytosis.

We have previously shown that actin severing by Ca^{2+}-activated gelsolin facilitates the binding step of collagen phagocytosis (Arora *et al*., 2004) and that collagen-induced Ca^{2+} fluxes occur only in the initial 200 s after contact with collagen (Arora *et al*., 2001). We show here that in the subsequent internalization step of phagocytosis, specific PIPKI isoforms generate PI(4,5)P2 after collagen binding; PI(4,5)P2 associates with nascent phagosomes and seems to be required for removal of gelsolin from actin filaments, thereby promoting actin assembly and collagen internalization. These data indicate that gelsolin can mediate discrete, sequential steps of collagen phagocytosis using distinct, actin-related functions.

MATERIALS AND METHODS

Reagents

Latex (2-μm-diameter) beads were purchased from Polysciences (Warrington, PA). Antibodies to β-actin (clone AC-15), bovine type 1 collagen (clone COL-1), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody, and tetramethylrhodamine B (RdB) isothiocyanate-phalloidin were from Sigma-Aldrich (St. Louis, MO). FITC-goat anti-rabbit antibody was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). The affinity-purifiedpolyclonal antibody to recombinant gelsolin has been described previously (Azuma *et al*., 1998). Profilin antibody (rabbit polyclonal) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Preparation

Fibroblasts were obtained from either wild-type or gelsolin null (Gsn^-) 12-d mouse fetuses as described previously (Witke *et al*., 1995). Cells were cultured in DMEM (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal calf serum and 10% antibiotics.

Collagen Bead Binding

Collagen-coated latex beads (2 μm) were applied to microbiological (i.e., nontissue culture) dishes and dried down for attachment as described previously (Arora *et al*., 2003) followed by washing with phosphate-buffered saline (PBS). The number of beads plated per dish was adjusted to produce final bead:cell ratios specific for each experiment. Cells were counted electronically, and the cell concentration was adjusted before plating cells on dishes containing collagen-coated beads. The plates were maintained at room temperature for 10 min to allow the cells to settle and subsequently washed with fresh medium at 37°C. Detached cells were removed by repeated washes. Those cells that were attached spread and rapidly internalized the collagen beads (Arora *et al*., 2000).

In experiments to evaluate collagen bead internalization, FITC-collagen-coated beads were incubated with cells for timed incubation periods. Internalization was stopped by cooling on ice. Fluorescence from the extracellular beads was quenched by trypan blue; internalized beads retained their bead-associated fluorescence (Arora *et al*., 2000). In some experiments, because gelsolin's severing activity enhances collagen bead binding (Arora *et al*., 2004), we expressed the data for bead internalization as percentage of beads internalized, thereby normalizing for the difference in the number of beads bound in Gsn^- cells.

However, immunolocalization of cells transfected with PIP kinase constructs requires fixation for antibody staining. Accordingly we developed a new method to differentiate internalized beads from cell surface-bound beads. Transfected Gsn^- or wild-type cells were incubated with FITC-streptavidin (40 μg/ml) for 30 min followed by a 1-h chase in α-minimal essential medium (MEM) and four washes with α-MEM (no serum). Samples were plated on biotinylated-collagen-coated beads and incubated for 20 min. Internalized beads were identified by the fluorescent green ring arising from the fusion of endosomes (labeled by FITC-streptavidin) with phagosomes (containing biotinylated-collagen-coated beads.

Severing Mutants

When creating actin filament severing mutants of gelsolin, the choice of altered residues was directed by the following considerations: the amino-terminal half of gelsolin is largely responsible for severing and capping activities and is regulated by phosphoinositides (Janmey and Stossel, 1987; Yin, 1987; Weeds and Maciver, 1993); in vitro severing assays have demonstrated that G1–3 and G2–6 sever filaments at 87 and 17% of the efficiency of wild-type gelsolin, respectively (Way *et al*., 1989). Accordingly, we targeted the high-affinity monomer actin binding sites in domain 1 and the F-actin side binding site in domain 2.

Previous studies have shown reduced binding affinity to actin filaments in RLK-210-AAA mutants (Puius *et al*., 2000), indicating a role for these residues as determinants of actin filament end and side binding. Our preliminary results with a RLK-210-AAA mutant exhibited 50% reduction in severing of actin filaments. Replacement of charged residues HR-119-EE and AAA-100-DDD alter actin binding but exert no effect on the structural stability of G1 (Way *et al*., 1992). Therefore, we created double mutants which included altered residues in both G1 and G2 (RLK-210-AAA/HE-119-EE and RLK-210-AAA/AAA-100-DDD. For design of control mutants, we examined gelsolin, adseverin, and villin sequences and selected nonconserved residues in domains 2 and 5, a strategy that would minimize the likelihood of mutating critical residues required for actin-dependent functions. In gelsolin domain 2, MLW-243-AAA, and in domain 5, QTA-530-AAA, were used as internal control mutants (Figure 2A).

Five different gelsolin mutants were created, and constructs were named by the mutated residues: GN.Wt, GN.210, GN.243, and GN.530 and double mutants GN.100/210 and GN.119/210. All mutations were confirmed by DNA sequencing. For production of mutant proteins, BL21 (DE3) cells (Novagen, Madison, WI) were transformed with wild-type or mutant gelsolin constructs. LB (250 ml) containing ampicillin (100 mg/ml) was inoculated overnight at 37°C followed by induction with isopropyl β-D-thiogalactoside (1 mM) for 4 h. Equivalent expression levels of the mutants was examined by immunoblotting. All gelsolin constructs were purified as described previously (Puius *et al*., 2000). Proteins isolated from inclusion bodies were dialyzed overnight and loaded onto glutathione-Sepharose 4B columns (GE Healthcare, Piscataway, NJ). The fusion proteins were cleaved on the column with thrombin (20 U in 2 ml of PBS) after overnight incubation. The eluate containing gelsolin mutants and thrombin were separated by Centricon 50 filters (Millipore, Billerica, MA). Protein yields were determined by optical density and protein analysis.

Selected sites were individually deleted by designing complementary primers and by the use of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Gelsolin mutants were ligated into BamH1 and Xho1 sites of the glutathione S-transferase (GST) fusion vector pGEX-4T-2 (GE Healthcare). The primers used were as follows: domain 2 forward (corresponding to amino acid residues 204–218), 5′-AACAGCAATCGGTATGAAGCTGCGGCTGCCACACAGGTGTCCAAGGG-3′ and domain 2 reverse, 5′-CCCTTGGACACCTGTGTGGCAGCCGCAGCTTCATACCGATTGCTGTT-3′. As described above, control mutations were also generated; the two sites selected were located in domain 2 (MLQ243AAA) and domain 5 (QTA530AAA) of mouse gelsolin. The primers used were as follows: domain 2 control forward (corresponding to amino acid residues 237–250), 5′-GGCACTGAGCCCGAGGCGGCTGCGGCTGTGCTGGGCCCCAAG-3′ and domain 2 control reverse, 5′-CTTGGGGCCCAGCACAGCCGCAGCCG-

CCTCGGGCTCAGTGCC-3′; and domain 5 control forward (corresponding to amino acid residues 524–538), 5′-ACCTCCCGCGAGGGCGGGGCTGCGGCTCCTGCCAGCAGCCGCCT-C-3′ and domain 5 control reverse, 5′-GAGGGCGGCTGCTGGCAGGAGCCGCAGCC-CCGCCCTCGCGGGAGGT-3′.

The complete cytoplasmic coding sequence for wild-type and mutated mouse gelsolin was subcloned into pGEX-4T-2 (GE Healthcare), pEGFP-C3 (BD Biosciences, San Jose, CA), and pDsRed2-C1 (BD Biosciences) using the following respective pair of primers: GEX forward, 5′-CCGCGTGGATCCCCAATGGTGGTGGAACACCCCG-3′ and GEX reverse, 5′-TGCGGCCGCTCGAGTGGCAGCCAGCTCAGCCAAGG-3′; GFP forward, 5′-TCAGATCTCGAGCTCATGGTGGTGGAACACCCCG-3′ and GFP reverse, 5′-AT-CCGGTGGATCCCGGGCAGCCAGCTCAGCCAAGG-3′; and DsRed forward, 5′-CTGAGATCTCGAGCTATGGTGGTGGAACACCCCG-3′ and DsRed reverse, 5′-TCCGGTGGATCCGGCAGCCAGCTCAGCCAAGG-3′.

Bead Incubation, Isolation, and Immunoprecipitation

Collagen-coated latex beads (6 μm) were attached to 100-mm nontissue culture plastic dishes. Cell suspensions were allowed to attach to beads for 20 min. Unattached, floating cells were aspirated and replaced with media warmed to 37°C to synchronize phagocytosis. In some experiments, collagen bead binding was examined at 4°C. Cells were collected at discrete time points thereafter. Cells and collagen-coated latex beads were collected with a cell scraper in extraction buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM Na_3VO_4, 20 μg/ml aprotonin, 1 μg/ml Pefabloc). The samples were sonicated (2 s) and centrifuged for 5 min at 8000 $\times$ *g* to remove unbroken cells. After clarification, equal amounts of proteins were incubated with antibodies to gelsolin, β-actin or PI(4,5)P2 to form immunocomplexes that were captured on Sepharose-G beads (Pierce Chemical, Rockford, IL) for 1 h at 4°C. The samples were boiled and separated on SDS-PAGE gels. Immunoblotted samples were probed with appropriate antibodies and quantified by scanning densitometry.

Phagosome Isolation and Actin Monomer Addition

De novo actin assembly in cells occurs extensively on membranes, including phagosomal membranes (Defacque *et al*., 2000). Previous studies have shown that after membrane-mediated actin nucleation, growth of filaments proceeds by insertion of new monomers adjacent to the membrane (Carlier, 1998). We studied directly the impact of the peptides PBP-10 and RhB-QRL (Cunningham *et al*., 2001) on actin assembly associated with phagosome formation. Phagosomes from Gsn$^-$ and wild-type cells were formed by incubation of cells (30 min) with 1-μm-diameter FITC-collagen-coated beads. Cells were pretreated with peptides before bead incubations. After incubations noninternalized beads were removed by extensive washing with PBS at 4°C. Cells were harvested by scraping into PBS and centrifuged at 1000 $\times$ *g* for 10 min, resuspended in buffer (20 mM HEPES, pH 7.0, 0.2 mM $CaCl_2$, 0.1 mM ATP), repelleted, suspended in 1 ml of the same buffer and lysed using 1-ml syringes fitted to 22-gauge needle. Nuclei and intact cells were removed by centrifugation at 800 $\times$ *g* at 4°C for 10 min. Prepared phagosomes were placed in polymerizing buffer (20 mM HEPES, pH 7.0, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM ATP, 0.03% fish gelatin) and protease inhibitors with 2 μM rhodamine-G-actin and 6 μM thymosin-β4 (Defacque *et al*., 2000). The phagosome mixture was placed on glass coverslips and incubated for 15 min at room temperature before observing by fluorescence microscopy. The integrity of phagosomes was confirmed by incubation with 0.2% trypan blue.

Actin monomer addition in permeabilized cells was performed as described previously (Arora *et al*., 2004). Briefly, cells were permeabilized for 20 s in 0.1 vol of OG buffer (PHEM buffer containing 2% octyl glucoside and 2 μM phalloidin). Permeabilization was stopped by diluting the detergent with buffer without detergent. Immediately thereafter, freshly sedimented rhodamine actin monomers (0.23 μM) in buffer containing 120 mM KCl, 2 mM $MgCl_2$, 3 mM EGTA, 10 mM PIPES, and 0.1 mM ATP were added to the samples for 10 s followed by fixation with 3.7% formaldehyde. The samples were observed with a Nikon TE 300 microscope. Rhodamine fluorescence in single cells was quantified using the PCI Imaging program. For estimation of background correction, detergent treatments were omitted, fluorescence was quantified, and this background signal was subtracted from experimental samples.

Circular Dichroism (CD)

For assessment of protein folding, CD spectra of gelsolin mutants were collected from 200 to 250 nm using a Jasco J-720 model spectrometer at 25°C. Spectra were scanned at 1-nm intervals, and three scans were averaged.

Severing and Capping Assays

Recombinant gelsolin mutant proteins were characterized by actin severing and capping assays. Protein concentrations were determined by the BCA protein assay kit (Pierce Chemical); equal amounts of gelsolin were used in all assays. The ability of gelsolin mutants (80–160 nM) to sever actin filaments was performed with pyrene-labeled actin (400 nM) at low calcium concentrations (0.01 mM). The ability of PI(4,5)P2 (30 μg/ml) to inhibit severing was performed by dispersing lipids for 15 s using sonication before addition to gelsolin and before addition to pyrene-labeled actin filaments. The reversal of inhibitory effect of PI(4,5)P2 (30 μg/ml) was determined by incubating with Triton X-100 (0.25%) before addition to gelsolin.

We measured the severing activity of lysates prepared from Gsn$^-$ cells that were previously transfected with mutant sequences cloned into the pcDNA3.1 vector. Cell lysates were collected with detergent plus protease inhibitors in buffer containing 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 2 mM Tris, pH 8.0, 1 mM EGTA, and 1% Triton X-100. Lysates were dialyzed with several changes of buffer B containing 2 mM $MgCl_2$, 50 mM KCl, 2 mM Tris-HCl, and 1 mM EGTA, 0.5 mM β-mercaptoethanol. The volume of the dialyzed cell lysate was adjusted to 400 μl in dialysis buffer. Pyrene-labeled actin filaments in polymerizing buffer (200 μl; 50 mM KCl, 2 mM $MgCl_2$) were added to a final concentration of 400 nM.

Immunofluorescence and Confocal Microscopy

Cells plated on beads were allowed to spread and bind to collagen beads for 30 min. Cells were fixed with 3% formaldehyde in PBS, permeabilized with 0.2% Triton X-100, and stained with polyclonal gelsolin antibody followed by FITC-tagged second antibody. The spatial distribution of gelsolin staining around beads was determined by confocal microscopy (Leica, Heidelberg, Germany; 40$\times$ oil immersion lens). Transverse optical sections were obtained at 1-μm nominal thickness.

Transfections and RNA Interference

To obtain more direct proof for a functional relationship between gelsolin expression and collagen phagocytosis in fibroblasts, Gsn$^-$ cells were transfected with a gelsolin expression vector. Gsn$^-$ cells were transfected using FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN). After titration experiments to determine the optimum concentration of vector, cells were transfected, incubated for 48 h and subjected to collagen bead binding assays.

PIPKIα and PIPKIγ661 siRNA oligonucleotides were synthesized using the following target sequences: AAGAAGTTGGAGCACTCTTGG and AAGGACCTGGACTTCATGCAG, respectively (Dharmacon, Lafayette, CO). 3′ Rhodamine sense strand-tagged GFP siRNA for the target sequence: CGGCAAGCTGACCCTGAAGTTCAT was purchased (QIAGEN, Mississauga, Ontario, Canada) as a control reagent. Mouse fibroblasts were seeded at 100,000 cells/35-mm tissue culture dishes and transfected with Oligofectamine reagent according to the manufacturer's instructions (Invitrogen). Experiments with PIPK1α- and PIPK1γ-silenced cells were performed 36 h posttransfection.

Statistical Analyses

For continuous variables, means and SEs of means were computed, and differences between groups were evaluated by Student's unpaired *t* test or ANOVA for multiple comparisons with statistical significance set at $p < 0.05$. Post hoc comparisons were performed with Tukey's test. For all experiments, at least three independent experiments were evaluated, each performed in triplicate. For analysis of actin severing and bead binding, regression analyses and Pearson correlation coefficients were computed to estimate loss of pyrene-actin fluorescence over time, and correlation coefficients were computed.

RESULTS

Disruptions of collagen phagocytosis cause imbalances in matrix homeostasis with important clinical consequences in tissues with rapid collagen turnover such as the periodontium (Everts *et al*., 1996). Accordingly, we examined the impact of gelsolin on the arrangement of collagen in histological sections of first molar periodontium from wild-type (wt) and gelsolin knockout mice (Gsn$^-$) (Figure 1A). Compared with the dense well-organized arrangement of collagen fibrils in wild type, sagittal sections of knockout mice showed irregular and poorly organized collagen as has been demonstrated in other models of disrupted collagen phagocytosis (Kataoka *et al*., 2000).

Role of Gelsolin during Collagen Bead Internalization

We showed previously a role for the severing function of gelsolin in enhancing collagen binding (Arora *et al*., 2004) and confirmed that the increased binding in gelsolin wild-type fibroblasts was not due to differences in surface collagen receptor expression ($\alpha2\beta1$ integrin). Because variations of collagen binding over time could also arise from differences of receptor turnover at 37°C between the Gsn$^-$ and wt cells, we measured binding at both 4 and 37°C. At 4°C,

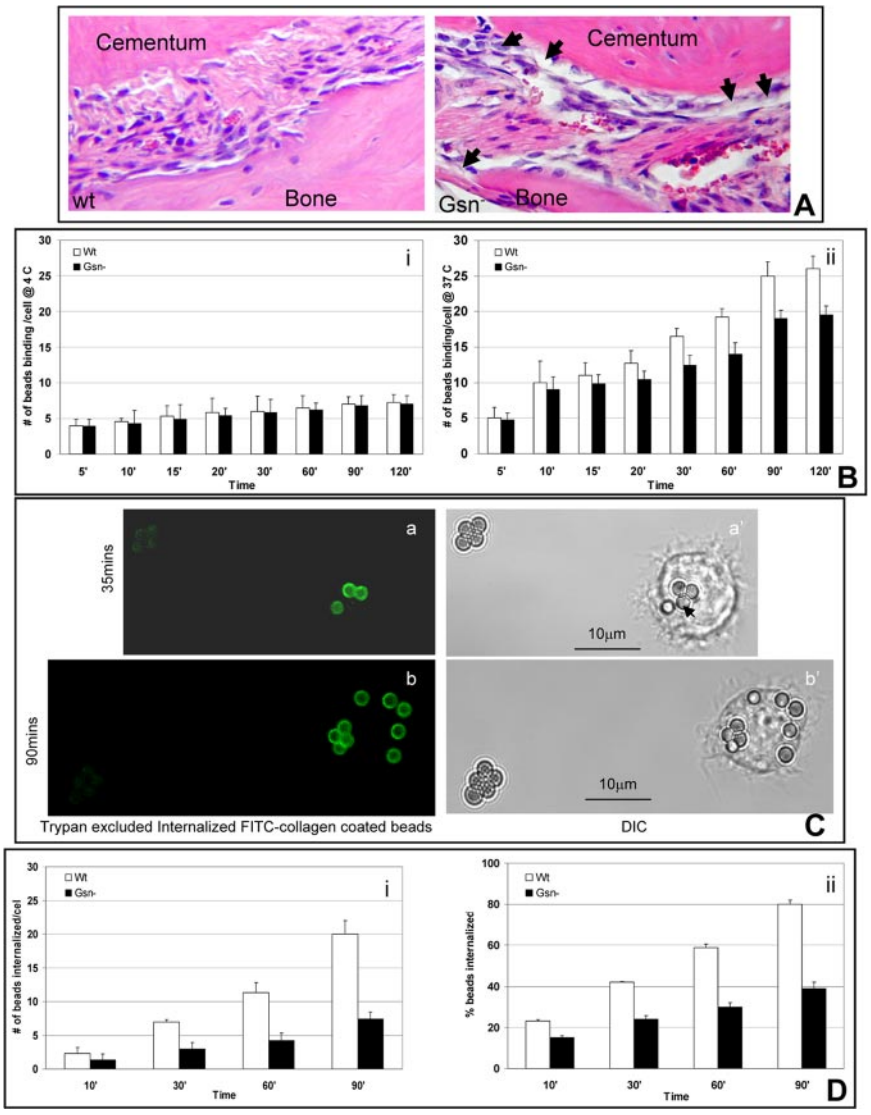

Figure 1. (A) Structure of molar periodontium. Sagittal paraffin sections through first molar periodontium from wt and Gsn⁻ mice were stained with hematoxylin and eosin to demonstrate organization of collagen in periodontal ligament. Note the poorly organized collagen in Gsn⁻ section (arrows). (B) Impact of gelsolin on collagen bead binding. Cells were incubated with FITC-collagen coated beads at 4°C (left) and 37°C (right). The number of bound beads was determined by fluorescence microscopy. Data (mean ± SEM) are representative of three independent experiments. (C) Collagen bead internalization. FITC-collagen coated beads were incubated with cells and at 35 min (a and a′) or 90 min (b and b′) internalization was stopped by cooling samples on ice. Fluorescence from noninternalized (i.e., extracellular) beads was quenched by trypan blue (shown by arrow). Internalized beads retained bead-associated fluorescence. (D) Impact of gelsolin on internalization. wt and Gsn⁻ cells were incubated with FITC-collagen beads for indicated times, and internalized beads were quantified by trypan blue quenching. Data (mean ± SEM) are representative of three independent experiments.

collagen binding increased slowly between 5 and 120 min, and there were no significant differences in bead binding between Gsn⁻ and wt cells. At 37°C, collagen bead binding increased ~2.3-fold in wt cells and ~1.8-fold in Gsn⁻ cells between 20 and 120 min (Figure 1B, i). Notably, Gsn⁻ cells exhibited significant collagen bead binding in spite of the absence of gelsolin (50–55% of wild type). At early time points (5–20 min), there were no significant differences in bead binding between Gsn⁻ and wt cells, indicating that basal collagen binding is equivalent.

To compare collagen internalization in Gsn⁻ and wt cells, cells were incubated with beads at 4°C for 15 min and subsequently washed and incubated with warm media (37°C) for varying time periods. Bound but not internalized FITC-collagen beads were distinguished from internalized beads by quenching with trypan blue. At 35 and 90 min after collagen bead incubation, samples treated with trypan blue exhibited fluorescence only for internalized beads (Figure 1C). In time-course experiments at 1 h, there was 2.7-fold higher collagen bead internalization in wt cells compared with Gsn⁻ cells (Figure 1D). Between 10 and 90 min, bead internalization increased nearly 8.7-fold in wt cells and 5.5-fold in Gsn⁻ cells, indicating significant differences in rates of collagen uptake between wt and gelsolin null cells.

Gelsolin Severing Mutants

Mutations of putative F-actin side binding residues in gelsolin domains G1 and G2 both cause reduced severing activity. Specifically, the following mutations in G1, HR-119-EE and AAA-100-DDD, and in G2, RLK-210-AAA, show altered actin binding but with no effect on the structural stability of their respective segments (Way *et al*., 1992; McGough *et al*., 1998; Puius *et al*., 2000). Our initial experiments with a full-length RLK-210-AAA mutant showed 50% reduction of severing activity. To reduce severing activity further, we created the double mutants RLK-210-AAA/HE-119-EE and RLK-210-AAA/AAA-100-DDD (GN.100/210). Nonconserved residues in G2, MLW-243-AAA, and in G5, QTA-530-AAA, were mutated and used as controls (Figure 2A). All mutations were confirmed by DNA sequencing. Bacterial cells (BL21 DE3) were transformed, and all proteins were expressed at equivalent levels for all mutants (Figure 2A). CD spectra of mutants (200–250 nm) collected at 25°C demonstrated no detectable alteration of the spectra, indicating

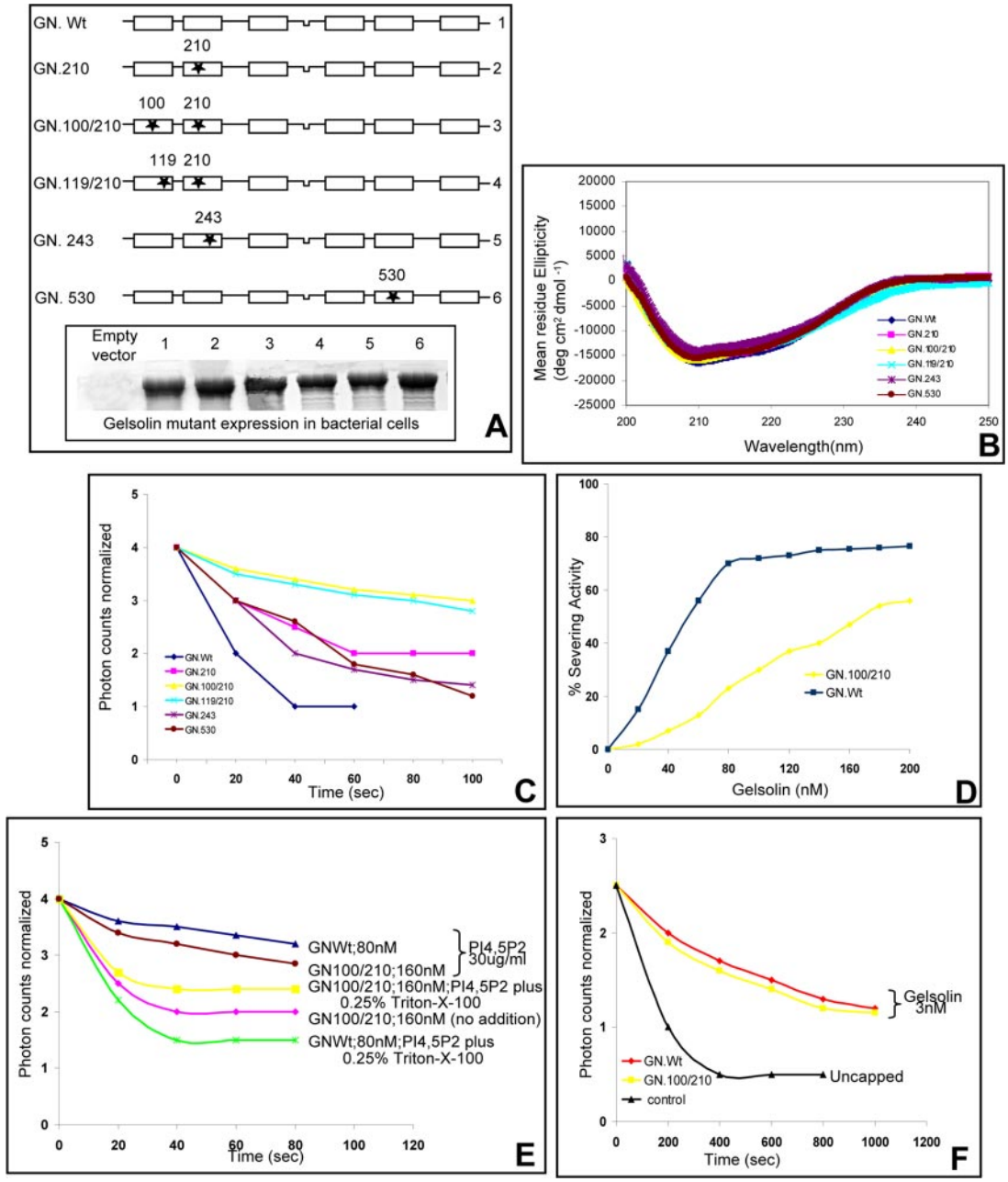

Figure 2. (A) Gelsolin mutants. Mutant proteins were designed as described in text. Transformed bacterial cells were immunoblotted to determine relative expression levels. (B) Protein conformation by circular dichroism. CD spectra of gelsolin mutants were collected from 200 to 250 nm at 25°C. Spectra were scanned at 1-nm intervals, and three scans were averaged. Note that mutants exhibit similar spectra as wild-type protein. (C and D) Fluorometric analysis of severing. (C) Pyrene-actin depolymerization assays were performed with 0.4 μM pyrene-labeled actin and 10 μM Ca^{2+}; gelsolin wild-type and mutant proteins were at 80 nM. The decrease in fluorescence was monitored and expressed in arbitrary units. GN.100/210, a double mutant shows 3.5-fold reduction in severing of actin filaments compared with wild-type gelsolin (GN.Wt). (D) Similar to GN.Wt the double mutant GN.100/210 exhibits concentration-dependent severing activity. (E) Inhibitory effect of PI(4,5)P2 on severing activities of mutants. F-Actin severing assays of gelsolin wild and mutant were performed with pyrene-labeled actin (400 nM) and PI(4,5)P2 (30 μg/ml). PI(4,5)P2 inhibited severing activity of the double mutant GN.100/210 (160 nM) and GN.Wt (80 nM). For the same samples, addition of 0.025% Triton X reversed PIP2-mediated inhibition of severing in GN.Wt and mutant samples. (F) Capping activity of gelsolin mutants. Wild-type and mutant gelsolins (3 nM) were compared for their ability to bind to the plus ends of actin filaments in the presence of 80 nM pyrene-F-actin and 10 μM Ca^{2+}. Gelsolin mutants prevented slow depolymerization of actin by capping barbed ends of actin filaments.

similar folding of the mutants as the wild-type gelsolin (Figure 2B). Mutants were analyzed for their ability to sever actin filaments. Pyrene actin depolymerization assays were performed in the presence of 0.4 μM labeled actin and 10 μM Ca^{2+}. Wild-type and mutant gelsolins were used at the same concentration (80 nM). GN.100/210, a double mutant, exhibited 3.5-fold lower actin severing activity than wild-type gelsolin (GN.Wt) and also showed slightly lower severing than

GN.210/119 (Figure 2C). Therefore, we selected the GN.100/210 mutant for further characterization and experiments.

GN.100/210 exhibited concentration-dependent severing activity, but there were fourfold and twofold lower severing activities between the mutant and wild-type gelsolins at 80 and 160 nM concentrations, respectively (Figure 2D). Incubation with PI(4,5)P2 (30 μg/ml) inhibited GN.Wt (80 nM) and GN.100/210 (160 nM) actin severing (Figure 2E). Disruption of micelles containing PI(4,5)P2 with 0.25% Triton X-100 preserved severing activity in GN.Wt and GN.100/210. Wild-type and mutant gelsolins (3 nM) were compared for their ability to bind to the plus ends of actin filaments in the presence of 80 nM pyrene-F-actin and 10 μM Ca^{2+}. The gelsolin mutant GN.100/210 capped the barbed ends of actin filaments as efficiently as GN.Wt (Figure 2F).

Severing Mutants Exhibit Reduced Collagen Binding but Not Internalization

Gsn^- fibroblasts were transfected with GN.Wt, GN.210, GN.100/210, or empty vector (pcDNA) (Figure 3A, a–d). Cell lysates were collected and processed as described in *Materials and Methods*. Double mutants showed ~20% of the severing activity of wild-type gelsolin, indicating significant residual severing activity that could mediate measurable collagen binding in the absence of gelsolin. We verified that equivalent amounts of gelsolin were present in all samples by immunoblotting lysates from transfected cells and probing for gelsolin (Figure 3A, e–h).

GFP-GN.Wt, GFP-GN.100/210, and GFP vector (control) were transfected into Gsn^- cells; transfected cells were harvested after 48 h and incubated on collagen-coated beads. There were no differences in the amount of de novo actin monomer addition at bead sites (10 min) in the gelsolin mutant (a–d) or wild-type (e–h) transfected cells (Figure 3B). Gsn^- cells transfected with empty vector exhibited monomer addition (~30% of wild-type), indicating that 30% of these processes were independent of gelsolin.

In Gsn^- cells transfected with the double gelsolin mutant, there was minimal increase in the numbers of collagen beads binding between 30 and 90 min ($p > 0.2$) whereas there was a nearly twofold increase in bead binding in cells transfected with the wt gelsolin ($p < 0.01$; Figure 3C, i). Over the same time interval, there were 1.5-fold ($p > 0.2$) and nearly threefold increases ($p < 0.01$) in the number of beads internalized in cells transfected with the mutant and the wild-type gelsolin, respectively. When we analyzed the time course data from 10 to 90 min by linear regression, for the mutant gelsolin the rate of bead binding was 0.04 beads/min and was 0.14 beads/min for the wt ($r^2 > 0.90$ for both cell types). The small, time-dependent increases of collagen binding and internalization in the cells transfected with the mutant gelsolin may be attributable to the residual actin severing activity of this construct (Figure 3, A–C). When we adjusted the raw bead internalization data for the difference in collagen bead binding between controls and mutant-transfected cells, there were no differences in bead internalization for all time points ($p > 0.2$; Figure 3C, iii). Collectively these data indicate that collagen bead binding is more dependent on the severing activity of gelsolin than is collagen internalization.

Association of Gelsolin with PI(4,5)P2

We determined whether PI(4,5)P2 was implicated in the regulation of collagen internalization. We first characterized the localization of PI(4,5)P2 in the binding and internalization steps of collagen phagocytosis. In time-course experiments, PI(4,5)P2 accumulation at collagen bead binding sites was inferred by transient transfection of a fusion protein consisting of the pleckstrin homology (PH) domain of PLCδ (which interacts with PI(4,5)P2; Stauffer and Meyer, 1997; Botelho *et al*., 2000) and GFP. Presumptive PI(4,5)P2 accumulation was detectable shortly after contact between the plasma membrane and collagen-coated beads (Figure 4A, a and b), and this accumulation was more pronounced around the phagocytic cup over time. To confirm that PI4,5P2 accumulation around beads was specifically due to collagen–integrin interactions, we examined the localization of PI4,5P2 in response to poly-L-lysine beads as these beads bind to fibroblasts but do not recruit integrins (Arora *et al*., 1995). We observed no accumulation of PI4,5P2 after bead contact at 5 and 10 min, although there was binding of the poly-L-lysine beads to cells (Figure 4B, a–c). Furthermore, we observed no internalization of poly-L-lysine beads (our unpublished data).

Phosphoinositide accumulation around collagen-coated beads was also studied by incubating viable cells with an electrostatically neutral complex of Bodipy FI PI(4,5)P2 in the presence of polybasic shuttle PIP carrier-2 (Molecular Probes, Eugene, OR), a reagent that facilitates probe transport into the cell. Confocal images were acquired over time and fluorescence intensities of line scans were used to quantify the extent of fluorescent PI(4,5)P2 accumulation. The fluorescence intensity around beads was corrected by subtracting the contralateral plasma membrane fluorescence and normalized to allow comparisons between cells. Results from the mean values of five different experiments showed that PI(4,5)P2 is spatially and temporally enriched around the beads as they are internalized (Figure 4C; 20 min). Only minimal fluorescence was detected around bound collagen beads at very early time periods (2 min).

We examined biochemical associations of gelsolin and PI(4,5)P2 after $\alpha 2\beta 1$ integrin engagement by collagen beads. We first determined whether increasing concentrations of PI(4,5)P2 (0.1–1.4 nM), when transferred to membranes, could be detected with PI(4,5)P2 antibody; under these conditions, we observed increased binding at higher concentrations of PI(4,5)P2 (Figure 4D, i). We next examined whether gelsolin associates with PI(4,5)P2 by immunoprecipitation as described previously (Azuma *et al*., 2000), a method that preserves the association between gelsolin and PI(4,5)P2. In cells stimulated with collagen beads, cell lysates immunoprecipitated with gelsolin antibody and then immunoblotted for $PI(4,5)P_2$ or anti-gelsolin antibodies showed time-dependent association of PI(4,5)P2 with gelsolin (Figure 4D, ii), which when quantified by densitometry, showed >6-fold increase between 0 and 10 min after collagen binding ($p < 0.01$; Figure 4D, iii). Cells transfected with GFP-PH(PLCδ), incubated with collagen beads and immunostained for gelsolin showed prominent colocalization of gelsolin and PI(4,5)P2 around beads (Figure 4D, iv, a–c).

Effect of Polyphosphoinositide Binding Peptides on Collagen Binding and Internalization

Actin assembly is an important requirement for particle internalization in macrophage phagocytosis (Aderem, 2003). Because the results mentioned above demonstrated increased gelsolin–PI(4,5)P2 interactions subsequent to collagen receptor engagement, we examined the possibility that PI(4,5)P2 mediates gelsolin-dependent collagen internalization. We disrupted gelsolin–PI(4,5)P2 interactions with a previously described, cell-permeant peptide (PBP-10: rhodamine B-QRLFQVKGRR) that corresponds to the sequence of the PI(4,5)P2 binding site of gelsolin (Cunningham *et al*., 2001). Initially, we determined whether PBP-10 and the control, QRL, interfered with severing in wild-type and Gsn^-

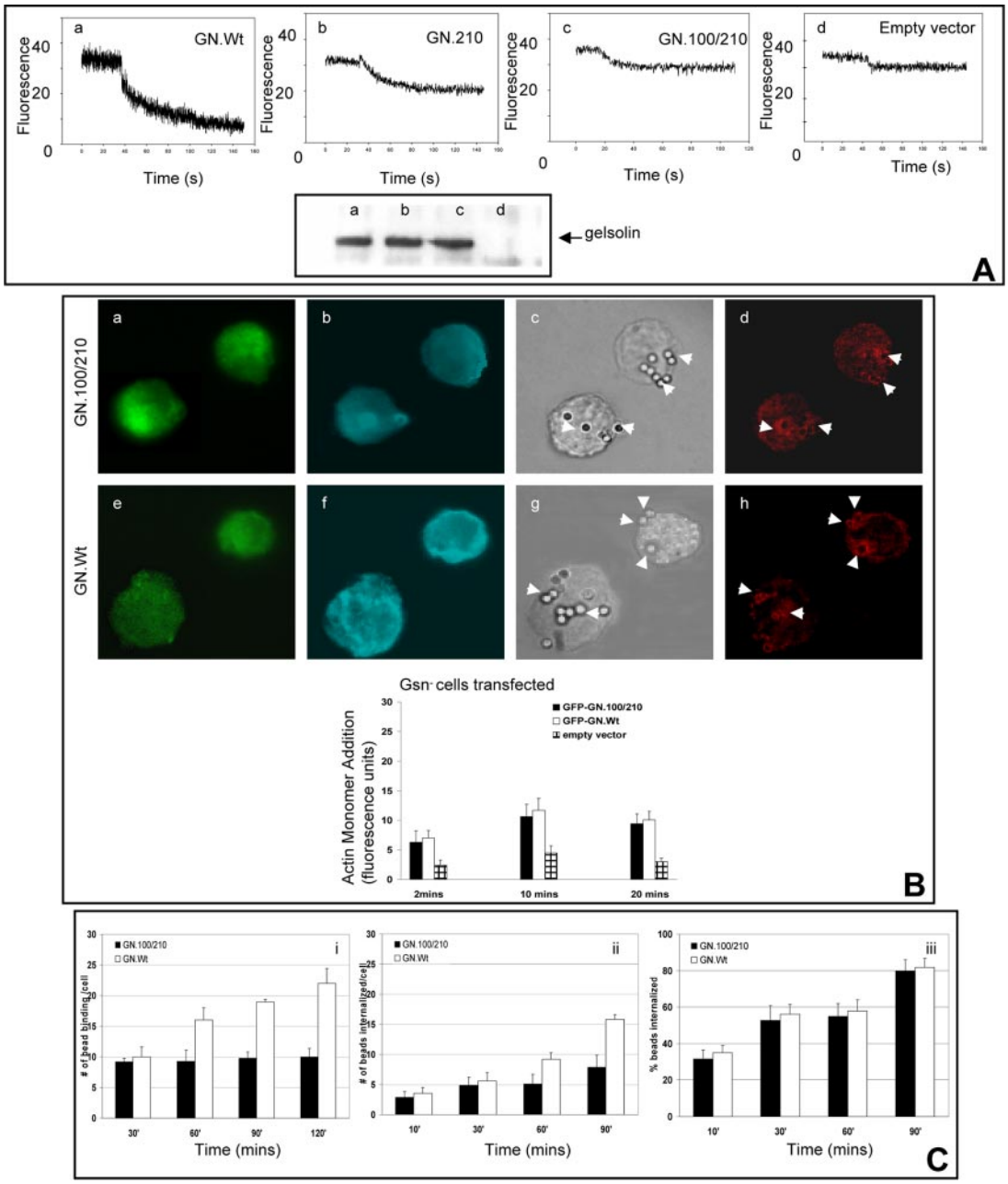

Figure 3. (A) GN.100/210-transfected cells show reduced severing activity. Cell lysates from Gsn$^-$ cells transfected with GN.Wt, GN.210, GN.100/210 or empty vector (a–d) were collected in buffer containing detergent plus protease inhibitors in buffer. Pyrene-labeled F-actin in polymerizing buffer (200 μl; 50 mM KCl, 2 mM $MgCl_2$) was added to a final concentration of 400 nM. Severing assays were performed in the presence of calcium (2 mM $CaCl_2$/1 mM EGTA). Note that double mutants show 20% of the severing activity of wild type. Bottom, to verify that equivalent amounts of gelsolin were present in all samples, lysates from cells transfected with different mutants, wild, and empty vector were immunoblotted (a–d) and probed for gelsolin. Protein concentrations were adjusted for each functional assay. For immunoblots, lane a, GN.Wt; lane b, GN.210; lane c, GN100/210; and lane d, empty vector. (B) Gsn$^-$ cells transfected with GFP-GN.100/210 show normal de novo actin monomer addition at collagen bead sites. GFP-GN.Wt, GFP-GN.100/210 and GFP-without vector were transfected into Gsn$^-$ cells. Transfected cells were harvested after 48 h and incubated on collagen-coated beads for 2, 10, and 20 min. Actin monomer incorporation in permeabilized cells was performed with rhodamine-labeled actin monomers. GFP (green) staining (a and e) shows transfected cells. Cells were stained with Alexa340-phalloidin for actin filaments (blue emission) (b and f). For background correction, detergent treatments were omitted, fluorescence was quantified, and this background signal was subtracted from experimental samples. Differential interference contrast images (c and g) and rhodamine actin (d and h). Rhodamine fluorescence around beads (arrows) was quantified from 25 transfected cells for each time point. For histogram at bottom, data are mean ± SEM of rhodamine actin fluorescence. (C) Gsn$^-$ cells transfected with DsRd-GN.100/210 show inhibition of collagen bead binding but minimal effect on bead internalization. After transfections (36 h) Gsn$^-$ cells were plated on FITC-collagen-coated beads for various time periods. At each time point, cells were placed on ice and extracellular fluorescence was quenched with 0.2% trypan blue for 5 min to distinguish internalized beads from beads on cell surfaces, followed by washing with PBS. Cells were quantified by fluorescence microscopy. For each time point, 50 cells were counted. Data (mean ± SEM) are representative of three independent experiments and are total number of beads bound and internalized counted in positively transfected cells.

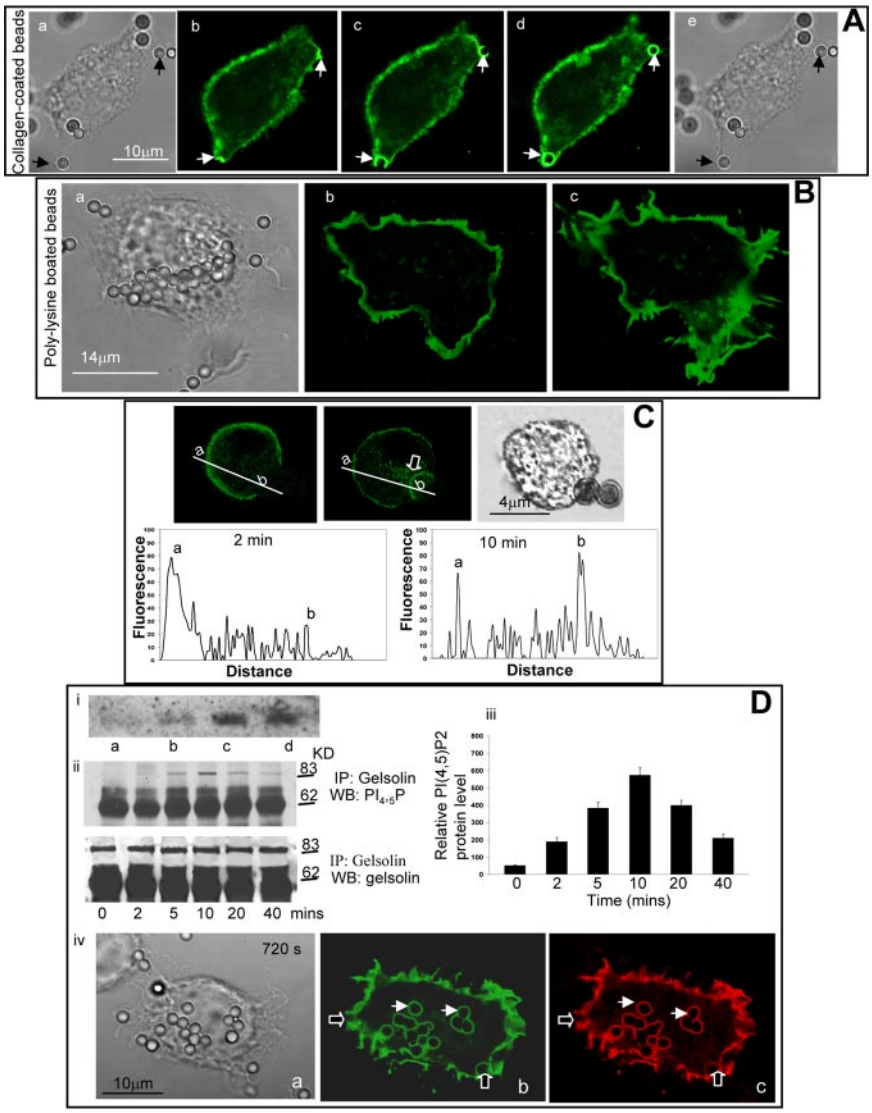

Figure 4. Collagen bead binding stimulates PI(4,5)P2 association with gelsolin. (A) Time course of PI(4,5)P2 accumulation at bead binding sites studied by transient transfection of a fusion protein consisting of PH domain of PLCδ and GFP. PI(4,5)P2 accumulation was detectable shortly after contact with collagen-coated beads (a and b). Accumulation around phagocytic cups increased over time (Figure 4A, c, 5 min; d and e, 10 min). (B) Confirmation that PI4,5P2 accumulation around beads was specifically due to collagen–integrin interactions from localization of PI4,5P2 in response to poly-L-lysine beads which bind nonspecifically to fibroblasts but are not internalized. (C) Phosphoinositide accumulation around collagen-coated beads studied with electrostatically neutral complex of Bodipy FI PI(4,5)P2 in presence of polybasic shuttle PIP carrier-2 to facilitate probe transport into cells. Confocal images were acquired over time. Fluorescence intensity of pixels at line scans were used to quantify extent of accumulation. Fluorescence intensities around beads were corrected by subtracting contralateral plasma membrane fluorescence and normalized to allow comparisons between cells. Results are representative of an average of five independent experiments and show maximal accumulation at ~10 min. (D) i, different concentrations of PI(4,5)P2 (0.1–1.4 nM) transferred to membranes were immunoblotted with PI(4,5)P2 antibody. (D) ii, biochemical interaction of gelsolin and PI(4,5)P2 examined by immunoprecipitation using antibody to gelsolin and immunoblotting with anti-PI(4,5)P2 or anti-gelsolin antibody respectively. Note that PI(4,5)P2 does not dissociate from gelsolin during SDS-PAGE and does not effect its migration. (D) iii, collagen bead binding stimulates PI(4,5)P2 association with gelsolin; maximal association at 10 min. (D) iv, GFP-PH(PLCδ) transfected cells after incubation with collagen beads were immunostained with antibody to gelsolin after 12 min. Note close association of gelsolin (red) with PI(4,5)P2 (green; a–c).

cells. Lysates from wild-type cells treated with PBP-10 and QRL peptide showed no difference in severing (PBP-10: −12.1 fluorescence units/s, $r^2 = 0.75$; QRL: −12.4 fluorescence units/s, $r^2 = 0.71$). Similarly, lysates from Gsn$^-$ cells treated with peptides also showed no difference of severing activity (PBP-10: −7.9 fluorescence units/s, $r^2 = 0.77$; QRL: −8.1 fluorescence units/s, $r^2 = 0.78$). As anticipated, there was much less severing activity detectable in the Gsn$^-$ cells.

Because PBP-10 binds to PI(4,5)P2 (Cunningham *et al*., 2001), it may reduce available PI(4,5)P2. Accordingly, we determined the localization of RdB-PBP-10 and the intracellular availability of PI(4,5)P2 in cells transfected with GFP-PH(PLCδ) and loaded with varying concentrations of the peptide (10–50 μM). Cells loaded with 30 μM RdB-PBP-10 showed diffuse cytoplasmic labeling (Figure 5A) and compared with untreated cells (Figure 4A), there was no effect on the subplasma membrane localization of PI(4,5)P2 as detected by GFP-PH(PLCδ) fluorescence. Cells treated with 40 μM peptide showed reduced staining of GFP-PH(PLCδ) at the plasma membrane, and at 50 μM, GFP-PH(PLCδ) fluorescence was markedly affected and was no longer present near the plasma membrane (Figure 5A). In subsequent experiments we used 30 μM RdB-PBP-10 or the control peptide at 30 μM.

We determined the effect of the peptides on collagen bead binding and internalization. Gelsolin wild-type cells were incubated for 10 min with the peptides (30 μM) and subsequently plated on FITC-labeled collagen-coated beads. At each time point, cells were incubated with trypan blue to help distinguish internalized beads from beads on the cell surface. PBP-10 had no effect on the number of collagen beads binding to the surface of cells compared with the control peptide. However, PBP-10 inhibited bead internalization by threefold compared with the QRL peptide ($p < 0.01$; Figure 5B, i), indicating that the collagen bead binding step is independent of phosphoinositide regulation but that collagen internalization requires phosphoinositide interaction with gelsolin. Compared with wild-type cells, gelsolin null cells show reduced collagen bead binding and bead internalization but there was no significant difference between PBP-10-treated and control peptide-treated cells, indicating that the threefold reduction of bead internalization we observed in the wild-type cells (Figure 5B, ii) is most likely due to interaction of PBP-10 with gelsolin and not

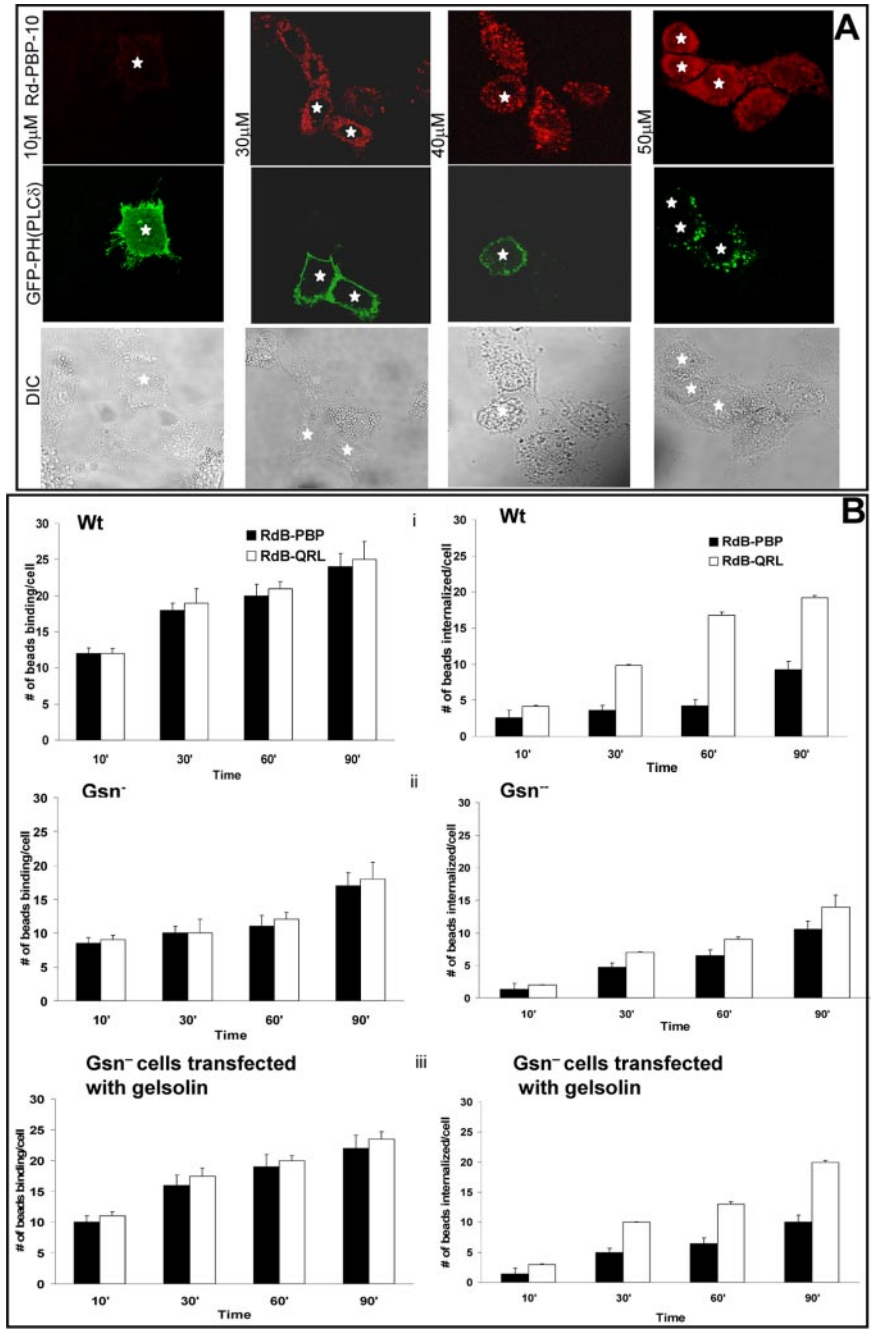

Figure 5. Effects of cell-permeant polyphosphoinositide binding peptides. (A) Intracellular distribution of different concentrations (10–50 μM) of RdB-PBP-10 peptide and interaction with PI(4,5)P2 in cells transfected with GFP-labeled fusion protein (PH domain of PLCδ). Note normal membrane-associated PI(4,5)P2 labeling in cells treated with lower concentration of peptide. (B) Cells (wt, Gsn$^-$, or Gsn$^-$ rescued with transfected gelsolin) were incubated for 10 min with peptides and then plated on FITC-labeled collagen-coated beads. At each time point, cells were treated with trypan blue to distinguish internalized beads from surface-bound beads. For each time point, 50 cells were counted. Data (mean $\pm$ SEM) are representative of three independent experiments.

because it is reducing availability of phosphatidylinositol bisphosphate (PIP2) that regulates other actin binding proteins.

To confirm the importance of PBP-10 in regulating collagen bead internalization, gelsolin null cells were transfected with GFP-gelsolin, sorted by flow cytometry into purified gelsolin-expressing cells, and these transfected cell were used for collagen bead binding and internalization assays. There was a twofold reduction in bead internalization in cells treated with PBP-10 but no impact on collagen binding (Figure 5B, iii)

Effect of PBP-10 on Actin Assembly

De novo actin assembly in cells frequently occurs on the cytoplasmic surface of membranes, including phagosomes (Defacque *et al*., 2000), and PI(4,5)P2 is known to regulate latex bead phagocytosis (Defacque *et al*., 2002). In vitro modeling of phagocytosis has been used to study how actin polymerization mediates the generation of force that drives plasma membrane extensions and helps to form lamellipodia, pseudopods, and phagosomes (Condeelis *et al*., 1988; Mitchison and Cramer, 1996; Defacque *et al*., 2000). We isolated collagen phagosomes and confirmed their integrity by incubation with trypan blue. Only phagosomes with intact membrane were included in analyses and only preparations with >70% intact phagosomes were assessed. For each experiment, incorporation of actin monomers was measured at the periphery of >50 phagosomes per treatment group. Rhodamine phalloidin-labeled actin filaments showed up as comet-like structures around the FITC-colla-

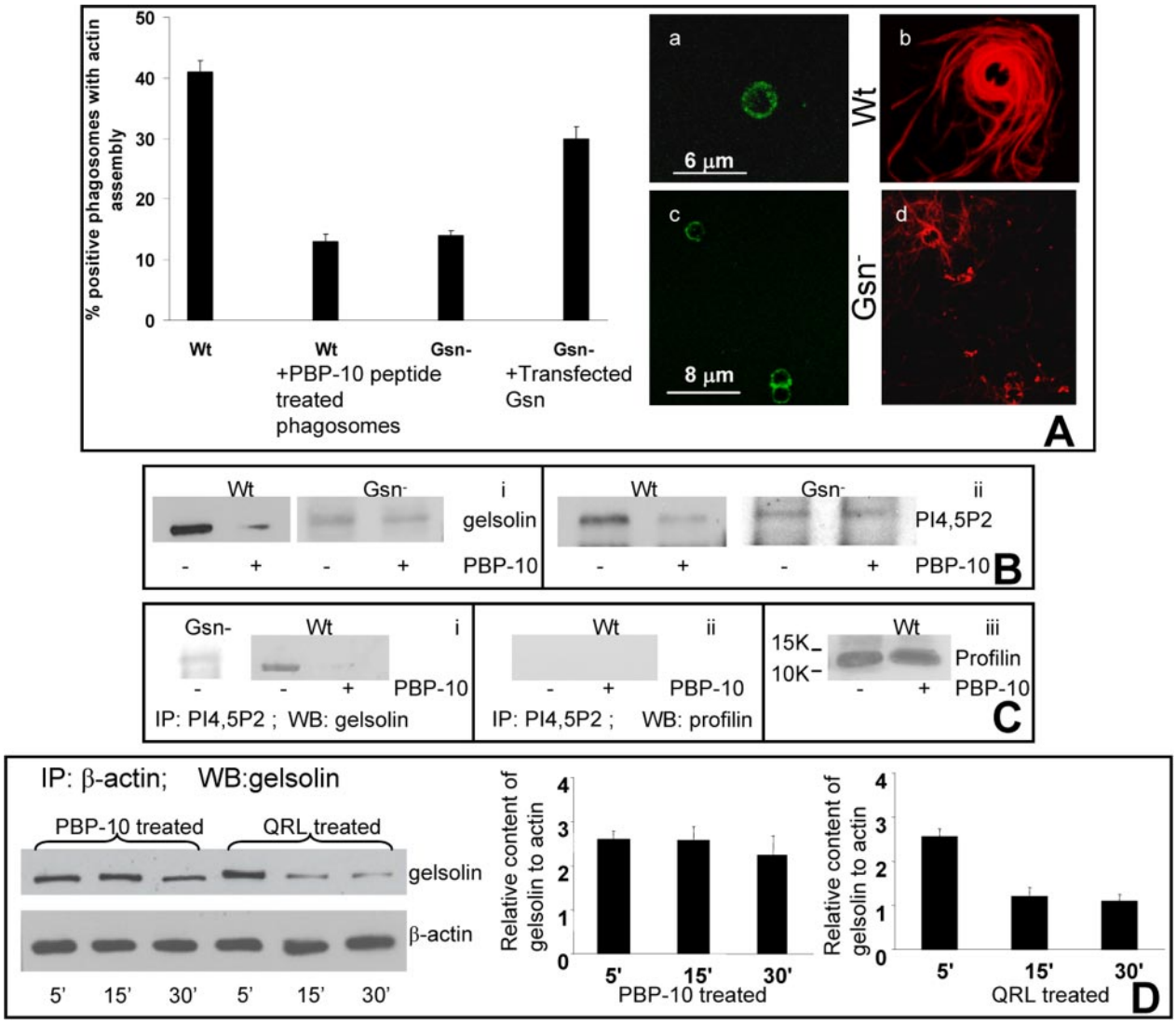

Figure 6. Effect of cell-permeant polyphosphoinositide binding peptides on actin assembly around collagen bead phagosomes. (A) Phagosomes formed in wt cells, or Gsn$^-$ cells, or Gsn$^-$ cells transfected with gelsolin. Cells were incubated with FITC-collagen-coated beads for 30 min. PBP-10 was incubated at 30 μM in wt cells. Phagosomes formed in vitro and actin filaments were stained with rhodamine phalloidin. For each experiment, 50 phagosomes were counted. wt cells consistently exhibited comet-like actin structures in a total of >250 cells, and these structures were not seen in the Gsn$^-$ cells. Data are mean ± SEM. (B) Phagosomes prepared from wt and Gsn$^-$ cells treated with and without PBP-10 peptide. Phagosomal proteins immunoblotted for gelsolin or PI(4,5)P2. (C) i, interaction of PI(4,5)P2 with gelsolin in wt and Gsn$^-$ cells examined by immunoprecipitation for PI(4,5)P2 and immunoblotting for gelsolin. PBP-10 peptide prevents gelsolin-PI(4,5)P2 interaction on phagosomes. (C) ii, interaction of PI(4,5)P2 with profilin on phagosomes examined by immunoprecipitation for PI(4,5)P2 and immunoblotting for profilin. (C) iii, no detectable reductions of profilin in phagosome-associated protein from PBP-10-treated or untreated samples. (D) Interaction of actin and gelsolin during phagocytosis. Cells were treated with PBP-10 or QRL control peptides. Equal number of phagosomes prepared at 5, 15, or 30 min after addition of collagen beads. β-Actin was immunoprecipitated from phagosomal proteins, and actin-bound gelsolin was immunoblotted. Note time-dependent reduction of gelsolin bound to actin in controls ($p < 0.01$), whereas PBP-10-treated samples show no change of gelsolin bound to actin over the same time interval ($p > 0.2$). β-actin was immunoblotted, showing equal amounts in each lane.

gen-coated bead-containing phagosomes in wt cells. We enumerated similar-looking structures in >250 wt cells that are different in appearance to the actin structures that grow from phagosomes isolated from macrophages (Defacque *et al*., 2000). These structures were not seen in the Gsn$^-$ cells. The PBP-10 peptide reduced the percentages of phagosomes showing actin assembly by >3-fold (Figure 6A). Gsn$^-$ cells transfected with cDNA gelsolin showed twofold higher numbers of phagosomes with actin assembly compared with Gsn$^-$ cells ($p < 0.01$; Figure 6A).

We examined the effect of the PBP-10 peptide on recruitment of gelsolin and PI(4,5)P2-bound gelsolin to phagosomes isolated from Gsn$^-$ cells and wt cells. Phagosome-associated proteins were immunoblotted [for gelsolin and PI(4,5)P2; Figure 6B, i and ii] or immunoprecipitated for PI(4,5)P2 and immunoblotted for gelsolin (Figure 6C, i). Wt cells treated with the peptide for 30 min showed greatly reduced amounts of phagosome-associated gelsolin and PI(4,5)P2. Furthermore, PI(4,5)P2-bound gelsolin was also eliminated by the peptide treatment, indicating that the

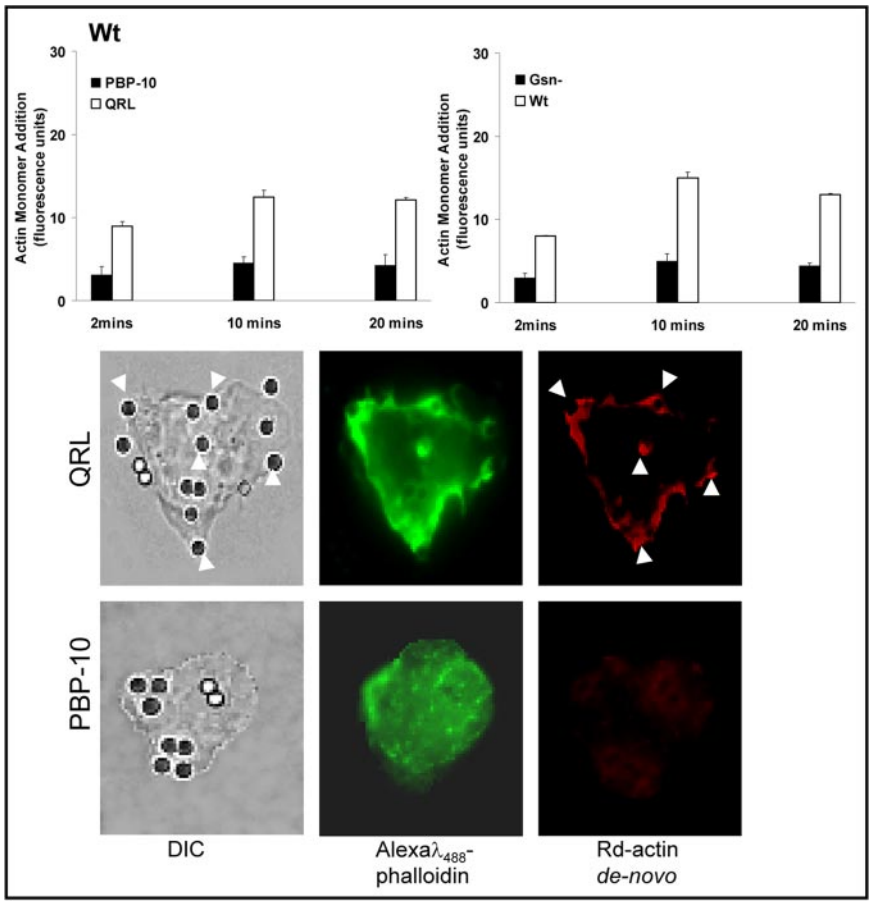

Figure 7. Effect of PBP-10 peptide on actin assembly at collagen bead phagocytosis sites. Direct examination of role of gelsolin by measurement of de novo incorporation of actin monomers into filaments around collagen-coated beads. Wild-type and Gsn$^-$ cells were incubated with collagen beads, permeabilized with OG, and incubated with rhodamine actin monomers. Note incorporation of actin monomers at bead sites (arrows) in wt cells treated with control peptide. Data are mean ± SEM of red fluorescence intensity in sampling area around phagosomes (n = 50 cells per treatment group).

PBP-10 peptide prevented the association of gelsolin with phagosomes and the interaction of PIP2 with gelsolin. In the absence of gelsolin (Gsn$^-$ cells), there was no detectable effect of PBP-10 on PI(4,5)P2 association with phagosomes.

In addition to gelsolin, PBP-10 could potentially interact with other actin binding proteins (e.g., profilin; Niggli, 2001). We examined the effect of PBP-10 on association of profilin with PI(4,5)P2. In immunoprecipitates for PI(4,5)P2 prepared from phagosomal proteins, there were undetectable levels of profilin in cells treated with or without the peptide (Figure 6C, ii). As expected, there were no detectable reductions of profilin in phagosomal proteins prepared from PBP-10 treated or untreated samples (Figure 6C, iii). These data indicate that gelsolin is a major phagosome-associated protein that is affected by the PBP-10 peptide.

If PI(4,5)P2 mediates the dissociation of gelsolin from capped actin filaments at phagosomes to create new barbed ends and promote actin assembly, we anticipated that at early time intervals (i.e., 5–30 min), treatment with the PBP-10 peptide would compete with gelsolin for available PI(4,5)P2. Furthermore, if PI(4,5)P2 is required for phagocytosis-induced dissociation of gelsolin from actin, we expected that the PBP-10 peptide would inhibit this process. We isolated phagosomes from cells treated with PBP-10 or the QRL-peptide at 5, 15, or 30 min after bead incubation. Phagosome-associated proteins were prepared from equivalent numbers of phagosomes, immunoprecipitated with β-actin antibody bound to protein G-Sepharose beads and immunoblotted for gelsolin. This procedure detected gelsolin bound to phagosomal actin and adjusted for the reduced amount of gelsolin (Figure 6B, i) and actin (Figure 7) that are associated with PBP-10-treated phagosomes. In samples treated with control peptide there were time-dependent reductions ($p < 0.01$; Figure 6D) of the relative amount of gelsolin bound to actin, indicating that in early stages of phagocytosis, gelsolin dissociates from actin filaments. In contrast, PBP-10-treated samples showed only small and insignificant changes over the same time interval ($p > 0.2$). β-Actin was adjusted to equal amounts in each sample to account for the reduced gelsolin that was present around the phagosomes in the PBP-10-treated samples. These findings indicated that the PBP-10 peptide blocked the dissociation of gelsolin from actin filaments and suggest that PI(4,5)P2 mediates the dissociation of gelsolin from capped actin filaments at phagosomes.

We determined more directly whether PI(4,5)P2 impacted phagosomal actin assembly. We first capped the barbed ends of actin filaments associated with phagosomes by incubation with recombinant full-length gelsolin (2 μM). Under these conditions, 18% of phagosomes (9 of 50) exhibited actin assembly as shown in Figure 6A. If these same preparations were treated with gelsolin, washed in EGTA (0.1 mM) buffer and subsequently incubated in PI(4,5)P2 (4 μM), 38% of phagosomes showed actin assembly (19 of 50). These data indicate that PI(4,5)P2 mediates actin assembly around collagen phagosomes.

Effect of Peptide on Actin Assembly at Bead-Cell Contact Sites

We measured the effect of the PBP peptide on de novo assembly of actin around the collagen-coated beads in intact cells. Cells (wt and Gsn$^-$) were incubated with collagen

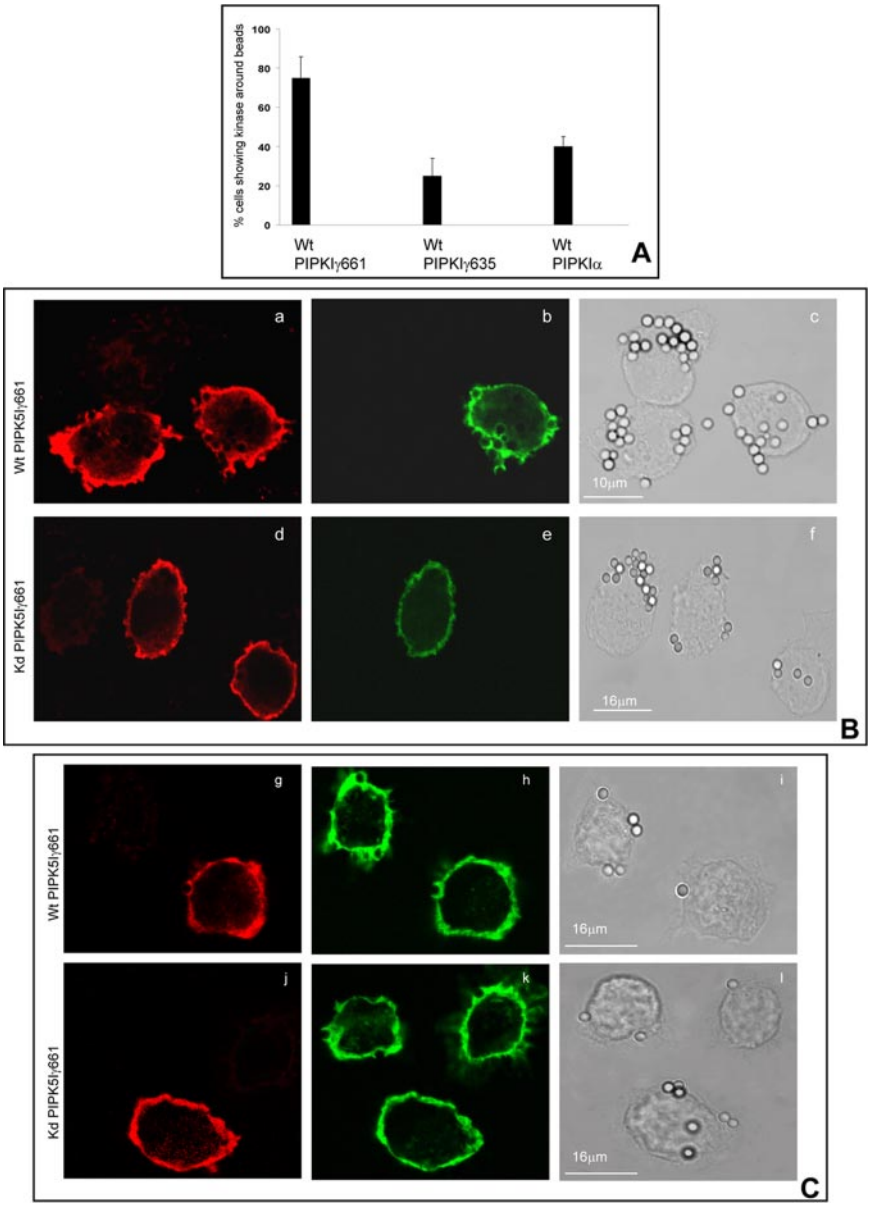

Figure 8. Catalytically inactive PIPK1γ661 impairs targeting around collagen-coated beads. (A) Cells were transiently transfected with different PIPK1 isoforms to study association with collagen-coated beads. Of 60 cells observed, 47 (78%) showed PIPK1γ661 accumulation around beads, 12 (20%) showed accumulation of PIPK1γ635, and 23 (38%) showed accumulation of PIPK1α. (B) Cells were cotransfected with GFP-PH(PLCδ) and wild-type/K_d PIPK1γ661. After 48 h, transfected cells were plated on collagen-coated beads for 10 min. Cells were fixed, permeabilized with 0.2% Triton X-100 and stained with anti-HA antibody followed by Cy3-conjugated second antibody to detect PIP kinase. Confocal microscopic sections show PI(4,5)P2 accumulation around collagen beads in cells transfected with wild-type PIPK1γ661 (b) samples but not in cells transfected with catalytically inactive mutant PIPK1 γ661 (e). (C) Compared with wild-type (g–i), accumulation of actin filaments in catalytically inactive PIPK1γ-transfected cells is reduced (j–l), and there is no phagosomal cup formation in the absence of kinase activity. In untransfected cells, cup formation occurs normally (h and k).

beads, permeabilized with octyl-glucoside, and incubated with rhodamine actin monomers. De novo actin assembly was observed around internalized beads over time. Cells treated with PBP-10 showed reduced incorporation of actin monomers that were similar to data obtained in Gsn^- cells (Figure 7), indicating that interaction of gelsolin with PI(4,5)P2 is a requirement for actin assembly in phagocytosis.

Role of PIPK1γ661 in Collagen-induced PI(4,5)P2 Formation

The data presented above demonstrate the importance of PI(4,5)P2-mediated regulation of gelsolin in collagen internalization through a mechanism involving actin assembly. We next investigated the role of PIP5K1 family members in terms of generation of PI(4,5)P2 at sites of collagen phagocytosis. Previous studies have implicated PIPK1α in macrophage phagocytosis (Coppolino *et al*., 2002), but our data showed minimal spatial association of PIPK1α with collagen-coated beads (Figure 8A). More recent studies have shown that type 1 phosphatidylinositol phosphate kinase isoform-γ661 (PIPK1γ661) is specifically targeted to focal adhesions and regulates synthesis of PI(4,5)P2 by its association with focal adhesion protein, talin. The interaction between PIPK1γ661 and talin may initiate focal adhesion assembly via regulation of β1-integrin binding by PI(4,5)P2 (Martel *et al*., 2001; Ling *et al*., 2002, 2003). PIPK1γ mRNA transcripts are alternatively spliced resulting in the expression of a PIPK1γ635 isoform that differs from the PIPK1γ661 isoform by a 26-amino acid carboxy-terminal extension. We transiently expressed PIPK1γ661 and PIPK1γ635 isoforms to study their association with collagen-coated beads. Of 60 cells examined, a >2-fold larger percentage of cells showed PIPK1γ661 accumulation around beads compared with PIPK1 γ635 ($p < 0.02$; Figure 8A).

We next analyzed the effect of wild-type and catalytically inactive PIPK1γ661 on the distribution of PI(4,5)P2 during early stages of phagocytosis. In cells cotransfected with wt

or catalytically inactive (K_d) PIPK1γ661 and GFP-PH(PLCδ), confocal sections showed colocalized PI(4,5)P2 and wild-type PIPK1γ661 accumulation around developing phagocytic cups adjacent to collagen beads. In contrast, cells transfected with the catalytically inactive kinase showed minimal labeling of PI(4,5)P2 to beads and incomplete phagocytic cup formation (Figure 8B).

Because actin filament assembly mediates extension of pseudopods around collagen beads, we examined actin filaments at phagosomal cups in cells transfected with the wild-type and catalytically inactive PIPK1γ661. Cells were fixed, permeabilized, and stained with Alexa488-phalloidin. Cells transfected with the catalytically inactive PIPK1γ661 showed markedly reduced amounts of actin filaments in the cups, whereas cells transfected with wild-type PIPK1γ661 or cells that were not transfected showed prominent actin staining (Figure 8C).

PIPKIγ661 Kinase Activity Mediates Collagen Bead Internalization

We determined whether PIPKIγ661 kinase activity affects collagen phagocytosis. Cells were plated on biotinylated-collagen-coated beads for 20 min; internalized beads were identified by the fluorescent green ring arising from the fusion of endosomes (FITC-streptavidin) with phagosomes (biotinylated-collagen-coated beads). In cells transfected with wild-type PIPK1λ635 or the catalytically inactive mutant, or with wild-type PIPK1α or the catalytically inactive mutant, there was no impact on bead internalization (Figure 9A). In contrast, transfection with catalytically inactive PIPK1γ661 caused threefold reductions of internalization ($p < 0.01$).

We used RNAi to reduce PIPK1γ661. Compared with untransfected cells or cells transfected with an irrelevant RNAi (GFP), the protein content of PIPK1γ661 was reduced by ~85% as shown in immunoblots and by immunostaining with peptide antibody to the C-terminal of PIPK1γ661 (Figure 9B, i and ii). Cells treated with RNAi PIPK1γ661 showed 2.5-fold reductions in bead internalization compared with untransfected or GFP RNAi-transfected cells (Figure 9B, iii).

Conceivably, low levels of PIPK1γ661 induced by RNAi PIPK1γ661 could affect surface expression of collagen receptors (α2β1 integrin), which in turn may have reduced collagen bead internalization. To assess whether cells treated with RNAi for PIPK1γ661 also exhibited reduced levels of surface-expressed collagen receptors, nonpermeabilized PIPK1γ661 or control (GFP) RNAi-treated cells were surface-labeled with FITC-labeled antibody to the α2β1 integrin and analyzed by flow cytometry. Cells treated with RNAi for PIPK1γ661 showed equivalent cell surface labeling of α2β1 as cells treated with GFP RNAi samples and mock-transfected cells (Figure 9B, iv).

DISCUSSION

Collagen phagocytosis is a critical process for extracellular matrix remodeling in physiological turnover and wound healing (Everts *et al*., 1996). Collagen phagocytosis is mediated predominantly by tissue fibroblasts, and, unlike the phagocytosis of microorganisms by professional phagocytes such as macrophages, is dependent on integrins. We defined here the role of gelsolin in collagen phagocytosis, an integrin-dependent process (Arora *et al*., 2000). We showed that two different activities of gelsolin, the severing of actin filaments and the PI(4,5)P2-mediated regulation of gelsolin, play temporally and functionally separate roles in two different stages of collagen phagocytosis binding and internalization.

We synthesized gelsolin severing deficient mutant, characterized their severing activity in transfected cells, and determined the importance of gelsolin's severing activity during the collagen internalization step of phagocytosis in fibroblasts. We also used PIP2 binding peptides that mimic the phosphoinositide binding region of gelsolin (Cunningham *et al*., 2001) and found that $PI(4,5)P_2$ is required for collagen internalization. Unlike the collagen binding step (Arora *et al*., 2004), internalization seems to be largely independent of gelsolin's severing function.

Dual Functions of Gelsolin in Phagocytosis

Previous subcloning and mutagenesis studies have used isolated gelsolin domains to examine in vitro the importance of specific residues in the actin filament binding sites of gelsolin and their impact on severing and capping (Way *et al*., 1989; Way *et al*., 1990, 1992; Sun *et al*., 1994; Burtnick *et al*., 1997; McGough *et al*., 1998; Puius *et al*., 2000). We selected two sites, one each in G1 and G2, to create gelsolin severing deficient mutants. We characterized the severing mutants in vitro using low calcium concentrations (10 μM in assay buffer) because G4–6 are calcium sensitive and enhance severing initiated by the calcium-insensitive domains (G1–3). For in vivo characterization, we subcloned mutants or wild-type gelsolin into different vectors that were then transfected into Gsn^- cells. Compared with cells transfected with wild-type gelsolin, cells transfected with severing mutants exhibited significantly reduced collagen binding, consistent with previous observations (Arora *et al*., 2004). Severing deficient mutants transfected into Gsn^- cells were fully capable of de novo recruitment of actin monomers at collagen binding sites, suggesting that generation of free barbed ends by gelsolin-mediated severing of actin filaments is not limiting for actin assembly in collagen phagocytosis.

Gsn^- cells transfected with empty vector exhibited monomer addition (~30% of wild type), indicating that 30% of these processes were independent of gelsolin. Evidently other actin binding proteins can promote de novo actin filament nucleation in response to integrin-induced phagocytosis (e.g., Arp 2/3; Wear and Cooper, 2004). In addition, Gsn^- cells exhibited measurable collagen binding and internalization, indicating that these processes are not wholly dependent on gelsolin. Nonetheless, both binding and internalization were markedly reduced in the Gsn^- cells compared with either wt cells or Gsn^- cells transfected with gelsolin. The major finding from binding and internalization data are that collagen bead internalization is much less affected by severing mutants than is collagen binding, indicating that gelsolin's severing function is relatively less important for membrane extension than is PI(4,5)P2-mediated uncapping.

Role of PIP2 in Collagen Phagocytosis

The localized generation of phosphoinositides [such as PI(4,5)P2)] can control dual functions of gelsolin, including the inhibition of gelsolin's severing activity and the uncapping of gelsolin from actin barbed ends (Sun *et al*., 1999). These processes can lead to localized bursts of actin nucleation and extension of plasma membranes by actin assembly. Our data demonstrate distinct spatiotemporal generation of PIP2 at sites of collagen bead internalization that is dependent on PIPK1γ661. Indeed, inhibition of PIPK1γ661 with dominant negative constructs blocked actin polymerization at sites of collagen bead internalization. Notably, focal adhesion formation depends on tyrosine phosphoryla-

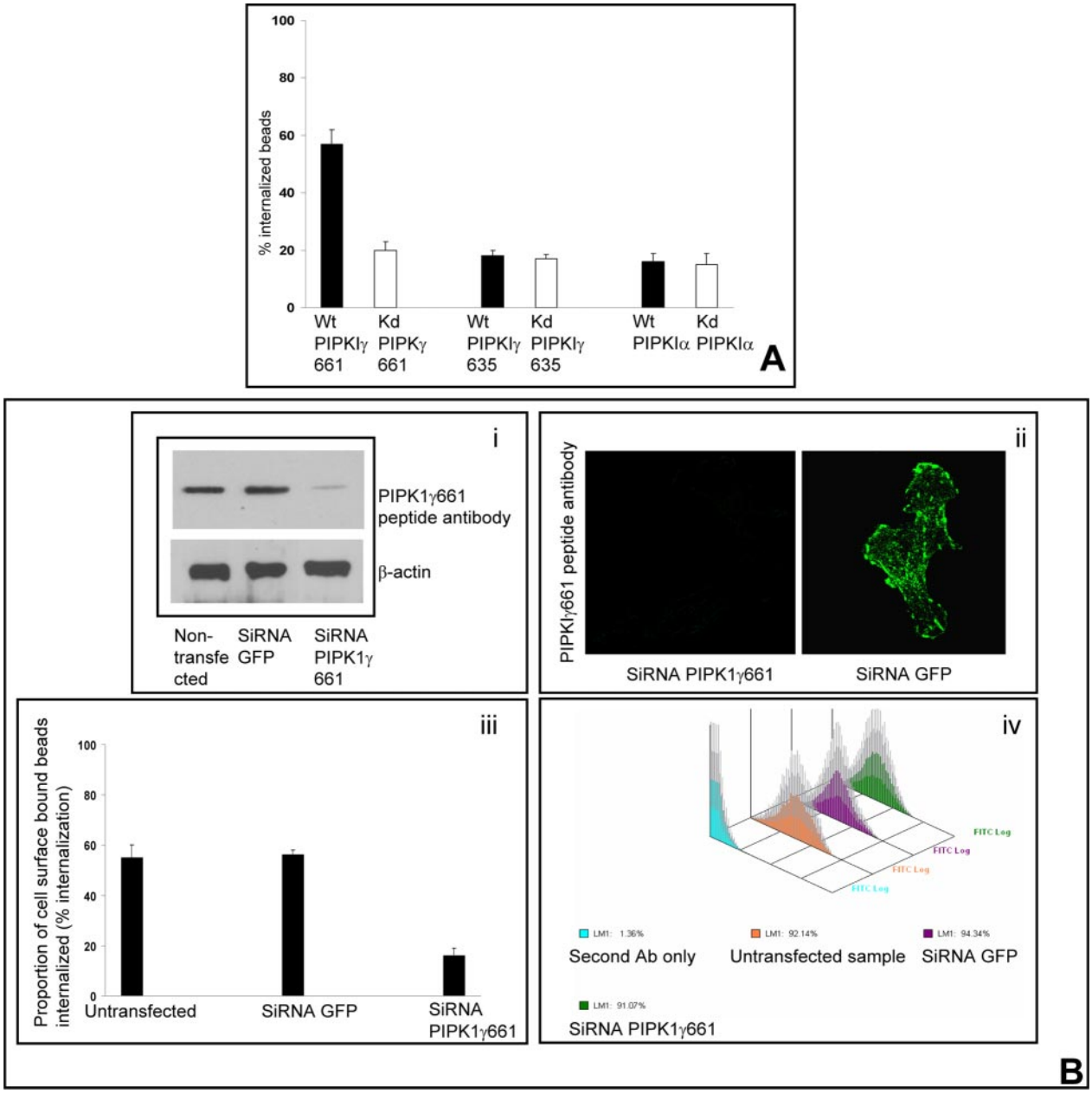

Figure 9. PIPK1γ661 kinase activity is required for collagen bead internalization. (A) Cells expressing wild-type PIPK1γ661 show threefold higher collagen bead internalization than cells transfected with catalytically inactive mutant (K_d). Cells transfected with the PIPK1γ635 isoform and the wild-type and catalytically inactive PIPK1α also showed reduced bead internalization. (B) i, in cells treated with RNAi for PIPK1γ661, immunoblotting with peptide antibody to C-terminal of PIPK1γ661 demonstrates extent of PIPK1γ661 knockdown. Individual samples were corrected for total protein concentration and coblotted for β-actin. GFP-RNAi and untransfected cells are controls. (B) ii, immunofluorescence analysis shows reduction of PIPK1γ661 staining in PIPK1γ661-RNAi-transfected cells. (B) iii, proportion of beads internalized was reduced by 2.5-fold in cells treated with PIPK1γ661 RNAi compared with untransfected control or GFP RNAi samples. (B) iv, flow cytometric analysis of surface labeling of α2β1 integrin in cells treated with PIPK1γ661 RNAi, GFP RNAi, or untransfected controls show no differences between samples.

tion of PIPK1γ661 and the generation of PI(4,5)P2, which facilitates the recruitment and regulation of talin and other focal adhesion proteins (Ling *et al*., 2002). Although PIPK1α is important in Fc-γ receptor phagocytosis in macrophages (Coppolino *et al*., 2002), our data show a specific requirement for the γ661 isoform (and not the α isoform of PIPK1) in collagen phagocytosis.

Our experiments with a cell-permeant, 10-residue peptide that competes with the PI(4,5)P2 binding region of gelsolin (Cunningham *et al*., 2001) markedly reduced collagen internalization. This effect was specifically due to interaction of the peptide with gelsolin because, in gelsolin null cells, the peptide did not block collagen internalization and did not affect localization of available $PI(4,5)P_2$. Conceivably, PBP-10 could competitively inhibit other PI(4,5)P2-regulated proteins such as profilin, but our studies here show that at least for profilin, there is no measurable impact on collagen phagosome binding.

We found that actin assembly on freshly isolated phagosomes and at collagen bead binding sites on cells was inhibited by the PBP-10 peptide. These data indicate that the peptide selectively and competitively blocks interactions of PI(4,5)P2 with endogenous gelsolin. This is a required step for uncapping actin filament ends and for increasing the availability of actin monomers for actin assembly at the collagen bead–integrin–actin interface (Hartwig *et al*., 1995; Glogauer *et al*., 2000). The demonstration that PI(4,5)P2 increases actin assembly around purified phagosomes and at collagen binding sites in whole cells further supports the notion that PI(4,5)P2-mediated removal of gelsolin from actin filaments is an important process for internalization. Previous work has shown that the gelsolin G1–3 domain stimulates actin assembly on phagosomes isolated from macrophages (Defacque *et al*., 2000). We observed the formation of comet-like actin filament structures around phagosomes, but only in the wt cells. In the Gsn^{-} cells, some actin assembly was noted around the phagosomes and these structures were morphologically closer in appearance to the structures reported previously (Defacque *et al*., 2000). Notably, the phagosomes in our current study were formed in fibroblasts in response to collagen receptor ligation (Arora *et al*., 2004) in contrast to the phagosomes formed in macrophages in response to latex beads (Defacque *et al*., 2000).

By immunoprecipitation to measure gelsolin–actin associations around collagen bead phagosomes, we found time-dependent dissociation of gelsolin from actin, a process that was inhibited by the PBP-10 peptide. Presumably, these data reflect PI(4,5)P2-mediated uncapping of actin filaments in the phagocytic process. These data are consistent with the notion that PI(4,5)P2, when synthesized in the vicinity of collagen phagosomes by PIPK1γ661, binds to gelsolin during the initial stages of actin assembly during collagen internalization and promotes uncapping. These results are in agreement with previous work showing that PIP kinase activity is required for PIP2–ezrin interactions and for actin polymerization (Defacque *et al*., 2002).

In addition to promoting the disassociation of gelsolin from actin filaments at sites of collagen internalization, PI(4,5)P2 may also sequester local pools of gelsolin by virtue of its binding site in G2 (Cunningham *et al*., 2001), thereby further inhibiting actin severing. Thus, the temporal regulation of gelsolin severing activity may be critical for efficient collagen internalization that relies on actin polymerization. Although we have demonstrated that PI(4,5)P2-may be involved in dissociation of gelsolin–actin complexes and is necessary for internalization, PI(4,5)P2 may also inhibit severing by gelsolin. In view of our previous demonstration of Ca^{2+}-dependent regulation of gelsolin severing and collagen binding (Arora *et al*., 2004), we conclude that separate, PI(4,5)P2-regulated functions of gelsolin may mediate discrete stages of collagen phagocytosis. We have provided data here implicating PI(4,5)P2-mediated dissociation of gelsolin from actin filaments around phagosomes as a mechanism for internalization but this notion is not definitively proven. Actin assembly on phagosomal membranes is a poorly understood and complex process and it is notable that some models of actin assembly on surfaces do not require uncapping (Dickinson and Purich, 2002).

ACKNOWLEDGMENTS

We thank David Kwiatkowski (Harvard University, Cambridge, MA) for provision of the gelsolin knockout mice, Arianna Roth for help with circular dichroism, and Cheung Lo for cell culture. The research was supported by Canadian Institutes of Health Research Group, Major Equipment and Operating Grants to C.A.M. and by a NORTH fellowship to M. C. We thank Sergio Grinstein for advice.

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